

Resistance to Colistin Associated with a Single Amino Acid Change in Protein PmrB among *Klebsiella pneumoniae* Isolates of Worldwide Origin

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A series of colistin-resistant *Klebsiella pneumoniae* isolates recovered from different countries was investigated in order to evaluate the involvement of the PmrA/PmrB two-component system in this resistance. Six isolates possessed a mutated PmrB protein, which is encoded by the *pmrB* gene, part of the *pmrCAB* operon involved in lipopolysaccharide modification. The same amino acid substitution (Thr157Pro) in PmrB was identified in the six isolates. The six isolates belonged to four distinct clonal groups, recovered in South Africa (sequence type 14 [ST14]), Turkey (ST101), and Colombia (ST258 and ST15). Three out of the four clones produced a carbapenemase, OXA-181, OXA-48, or KPC-3, while a single isolate did not produce any carbapenemase. Expression assays revealed an overexpression of the *pmrA* (70-fold), *pmrB* (70-fold), *pmrC* (170-fold), and *pmrK* (40-fold) genes in the *pmrB*-mutated isolate compared to expression of the *pmrB* wild-type isogenic *K. pneumoniae* isolate, confirming that the PmrB substitution was responsible for increased expression levels of those genes. Complementation assays leading to the expression of a wild-type PmrB protein restored the susceptibility to colistin in all isolates, confirming that the substitution in PmrB was responsible for the resistance phenotype. This study identified a key amino acid located in the PmrB protein as being responsible for the overexpression of *pmrCAB* and *pmrHFIJKLM* operons, leading to resistance to colistin.

Klebsiella pneumoniae is a Gram-negative pathogen often associated with nosocomial infections, including pneumonia, bacteremia, urinary tract infection, and sometimes even life-threatening septic shock (1). Infection caused by multidrug-resistant (MDR) *K. pneumoniae* is now a worldwide issue, considering the wide dissemination of MDR clones. In particular, the recent worldwide emergence of carbapenemase-producing isolates has often resulted in very limited therapeutic options. As a consequence, polymyxin antibiotics are now considered the last resort in the armamentarium for treatment of infections caused by MDR Gram-negative pathogens.

Polymyxins are derivatives of the *Bacillus polymyxa* subspecies *colistinus* that are active only against Gram-negative bacteria. Structurally, they are decapeptides bound to a fatty acid chain (2). The clinically available forms, polymyxin B and colistin (also known as polymyxin E), are administered intravenously or by inhalation. Like other antimicrobial peptides (APs), colistin interacts with the lipid A moiety of the Gram-negative bacterial lipopolysaccharide (LPS). The polycationic peptide ring competes for and substitutes the calcium and magnesium bridges stabilizing the LPS, promoting membrane permeability and thus disrupting the integrity of the outer membrane of Gram-negative bacteria, thus leading to bacterial death (2).

Nevertheless, resistance to polymyxins has been reported in *K. pneumoniae*, but little is known about the molecular mechanisms sustaining this resistance trait. The two-component regulatory systems (TCS) PmrA/PmrB and PhoP/PhoQ have been identified as regulatory systems involved in the resistance to polymyxin B (3). The insertional inactivation of the PhoQ/PhoP *mgrB*-encoding regulator has also been recently associated with colistin resistance (4, 5).

The main mechanisms leading to resistance to polymyxins are modifications of the bacterial outer membrane, mainly through the covalent addition of both phosphoethanolamine (pEtN) and 4-deoxyaminoarabinose (Lara4N) to the LPS (6–8). The *pmrCAB* operon encodes the PmrC phosphoethanolamine phosphotransferase, the PmrA response regulator (also named BasR), and the PmrB sensor kinase (also named BasS) (6). In *Escherichia coli* and *Salmonella enterica* species, PmrB acts as a sensor cytoplasmic membrane-bound kinase activated by high concentrations of iron (Fe³⁺) and an acidic pH (pH 5.5). Upon activation, it activates PmrA by phosphorylation (2). *In vivo*, PmrAB is involved in sensing the environment and is required for intramacrophage survival and virulence (6). Mutations in the *pmrA* or *pmrB* genes usually result in constitutive activation of PmrA, which upregulates in turn three loci, namely, *pmrC* encoding an aminotransferase involved in the decoration of the LPS with pEtN, *pmrE* (previously identified as *pagA* or *ugd*) encoding a UDP-glucose dehydrogenase, which is the first enzyme in the Ara4N biosynthetic pathway, and the *pmrHFIJKLM* operon (also called *pmrF*, *pbg*, or *arn* operon) encoding enzymes responsible for the synthesis and

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transfer of the Lara4N to lipid A (6). Overall, this constitutive activation of PmrA leads to a more positively charged LPS, thus reducing the affinity of positively charged polymyxins.

Using a collection of *K. pneumoniae* isolates recovered worldwide, in this study we aimed to investigate whether the mechanism leading to resistance to colistin might be related to the PmrAB two-component system.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Thirty-five colistin-resistant *K. pneumoniae* clinical isolates were included in this study. They had been collected in France, Turkey, Colombia, and South Africa. Isolates were identified by using the API20E system (bioMérieux, Marcy l'Etoile, France). *E. coli* TOP10 (Invitrogen, Illkirch, France) was used as the host strain for cloning experiments with selection based on kanamycin (50 µg/ml). Colistin-resistant *K. pneumoniae* clinical isolates were also used for transformation assays and selection based on zeocin (a formulation of phleomycin D1, a glycopeptide antibiotic produced by *Streptomyces verticillus*) (100 µg/ml).

Antimicrobial susceptibility assays. The antibiotic susceptibility testing was performed using Etest strips (AB bioMérieux, La Balme-les-Grottes, France) on Mueller-Hinton agar plates (Bio-Rad, Marnes-la-Coquette, France) with 0.5 McFarland standard inoculum. MICs were also determined by broth microdilution in cation-adjusted Mueller-Hinton broth (MHB-CA) according to CLSI guidelines (9–11). Polymyxin B and colistin (Sigma-Aldrich, Saint-Quentin Fallavier, France) were tested with Tween 80 (a surfactant preventing binding of colistin to drug panels) over a range of dilution from 0.125 to 64 µg/ml (9, 12). Briefly, MHB-CA with a final concentration of 0.002% Tween 80 and 5×10^5 CFU/ml in each well was used. Following the EUCAST breakpoints (<http://www.eucast.org/>), isolates with a colistin MIC of ≤ 2 µg/ml were categorized as susceptible, although those with MICs of > 2 µg/ml were resistant.

PCR amplification and sequencing. The chromosomal DNA was isolated using the commercially available QIAquick kit (Qiagen, Courtaboeuf, France), according to the manufacturer's instructions. The *pmrA*, *pmrB*, *phoP*, *phoQ*, and *mgrB* genes possibly involved in resistance to colistin were amplified using specific oligonucleotides (Table 1). The PCR amplification for detection of the narrow- and expanded-spectrum β -lactamase genes *bla*_{CTX-M}, *bla*_{SHV}, *bla*_{TEM}, *bla*_{OXA}, *bla*_{OXA-48-like}, and *bla*_{KPC} was carried out as described previously (13, 14). The amplified DNA fragments were purified with the QIAquick PCR purification kit (Qiagen). Both strands of the amplification products obtained were sequenced with an ABI 3100 sequencer (Applied Biosystems, Foster City, CA). The nucleotide and deduced protein sequences were analyzed at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov) by the Basic Local Alignment Search Tool (BLAST) program.

Analysis of the primary and secondary structures of the PmrB protein. The primary structure of the PmrB protein was analyzed using the Ensembl Bacteria database (<http://bacteria.ensembl.org/index.html>). The predicted secondary structures of the two PmrB proteins (wild-type and mutated) were obtained using the GOR method with EMBOSS 6.3.1 software, available on the Mobyle@Pasteur portal (<http://mobyle.pasteur.fr/cgi-bin/portal.py>).

Complementation assays. The *pmrB* gene from the *K. pneumoniae* reference strain ATCC 53153 (colistin MIC of 0.125 µg/ml) was amplified by PCR using 2× Phusion HF master mix (Finnzymes; Life Technologies, Illkirch, France) and primers *pmrB* ext F and *pmrB* ext R. The partial and therefore noncoding *mdh* sequence (supposed to encode a malate dehydrogenase [15]) was PCR amplified with primers *mdh* ext F and *mdh* ext R (Table 1). Using the Zero Blunt TOPO PCR cloning kit (Invitrogen), the amplified fragments were cloned into the high-copy-number plasmid pCR-BluntII-TOPO encoding resistance to kanamycin and zeocin. The resulting plasmids pTOPO-*pmrB* and pTOPO-*mdh* were separately transformed into electrocompetent *E. coli* TOP10 strains by electroporation. Transformants were selected by overnight incubation at 37°C on Mueller-

TABLE 1 Oligonucleotides used in this study

Primer	Sequence (5' to 3')	Gene	Reference or source
<i>pmrA</i> ext F	CAT TTC CGC GCA CTG TCT GC	<i>pmrA</i>	This study
<i>pmrA</i> ext R	CAG CTT TCA GTT GCA AAC AG	<i>pmrA</i>	This study
<i>pmrB</i> ext F	ACC TAC GCG AAA AGA TTG GC	<i>pmrB</i>	This study
<i>pmrB</i> ext R	GAT GAG GAT AGC GCC CAT GC	<i>pmrB</i>	This study
<i>phoP</i> ext F	GAG CTT CAG ACT ACT ATC GA	<i>phoP</i>	This study
<i>phoP</i> ext R	GGG AAG ATA TGC CGC AAC AG	<i>phoP</i>	This study
<i>phoQ</i> ext F	ATA CCC ACA GGA CGT CAT CA	<i>phoQ</i>	This study
<i>phoQ</i> ext R	CAG GTG TCT GAC AGG GAT TA	<i>phoQ</i>	This study
<i>mgrB</i> ext F	TTA AGA AGG CCG TGC TAT CC	<i>mgrB</i>	4
<i>mgrB</i> ext R	AAG GCG TTC ATT CTA CCA CC	<i>mgrB</i>	4
<i>mdh</i> ext F	CCC AAC TCG CTT CAG GTT CAG	<i>mdh</i>	15
<i>mdh</i> ext R	CCG TTT TTC CCC AGC AGC AG	<i>mdh</i>	15
<i>pmrC</i> int F	GCG TGA TGA ATA TCC TCA CCA	<i>pmrC</i>	This study
<i>pmrC</i> int R	CAC GCC AAA GTT CCA GAT GA	<i>pmrC</i>	This study
<i>pmrA</i> int F	GAT GAA GAC GGG CTG CAT TT	<i>pmrA</i>	This study
<i>pmrA</i> int R	ACC GCT AAT GCG ATC CTC AA	<i>pmrA</i>	This study
<i>pmrB</i> int F	TGC CAG CTG ATA AGC GTC TT	<i>pmrB</i>	This study
<i>pmrB</i> int R	TTC TGG TTG TTG TGC CCT TC	<i>pmrB</i>	This study
<i>pmrD</i> int F	GAT CGC AGA GAT TGA AGC CT	<i>pmrD</i>	This study
<i>pmrD</i> int R	GCG TTG CGG ATC TTC AAA GT	<i>pmrD</i>	This study
<i>pmrE</i> int F	GCA TAC CGT AAT GCC GAC TA	<i>pmrE</i>	This study
<i>pmrE</i> int R	GGG TTG ATC TCT GTG ACA TC	<i>pmrE</i>	This study
<i>pmrK</i> int F	AGT ATC GGT CAG TGG CTG TT	<i>pmrK</i>	This study
<i>pmrK</i> int R	CCG CTT ATC ACG AAA GAT CC	<i>pmrK</i>	This study
<i>rpsL</i> int F	CCG TGG CGG TCG TGT TAA AGA	<i>rpsL</i>	4
<i>rpsL</i> int R	GCC GTA CTT GGA GCG AGC CTG	<i>rpsL</i>	4

Hinton agar supplemented with kanamycin (50 µg/ml). Plasmids were then isolated using the QIAprep spin miniprep column kit (Qiagen) and transformed into colistin-resistant *K. pneumoniae* clinical isolates. Transformants were selected by overnight incubation at 37°C on Mueller-Hinton agar supplemented with zeocin (100 µg/ml), and the presence of the cloned gene was checked by PCR. The MICs of transformants for colistin and polymyxin were determined as described above.

Strain genotyping by PFGE and MLST. The genetic relatedness of the isolates was assessed by pulsed-field gel electrophoresis (PFGE) analysis with XbaI-digested genomic DNA as described previously (16). Multilocus sequence typing (MLST) was carried out with seven standard housekeeping loci (*rpoB*, *gapA*, *mdh*, *pgi*, *phoE*, *infB*, and *tonB*) according to Diancourt et al. (15). Sequence types were analyzed by using the Institut Pasteur database (<http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html>).

Growth curves. In order to evaluate the putative impact of modified LPS, growth curves were determined. Briefly, 200 ml Luria broth was inoculated independently with 10^8 CFU of each strain, and cultures were grown for 24 h at 37°C under gentle shaking. Absorbance at a wavelength of 600 nm was measured during 24 h. The colony counting was performed by serial dilution and final plating on solid medium. Experiments were repeated three times. Zeocin (100 µg/ml) was added to the growth medium in order to select the recombinant plasmids when *K. pneumoniae* strain AF1b complemented with pTOPO-*pmrB* or pTOPO-*mdh* was used.

Transcriptional analysis by quantitative real-time PCR. Quantitative real-time PCR (qRT-PCR) was used to measure the expression of the *pmrC*, *pmrA*, *pmrB*, *pmrD*, *pmrE*, and *pmrK* genes, using the primers listed in Table 1. RNA preparations were obtained using the RNeasy minikit (Qiagen), according to the manufacturer's instructions. qRT-PCR was carried out using a Rotor-Gene Q instrument (Qiagen). Expression of the *rpsL* gene encoding a ribosomal protein was evaluated with the primers *rpsL* int F and *rpsL* int R (Table 1), and this expression rate was used as a signal reporter (4). QuantiFast SYBR green (Qiagen) was used as an in-

TABLE 2 MICs, molecular features, and genotyping analysis of *K. pneumoniae* isolates carrying the *pmrB* gene mutation and of isolate AF1a, the isogenic colistin-susceptible counterpart of AF1b

Isolate	Country of isolation	MIC ($\mu\text{g/ml}$) for ^a :					Colistin profile ^b	PmrB amino acid change	Carbapenemase	ESBL ^c	Additional β -lactamases	MLST ^d	PFGE ^e pulsotype
		ERT	IPM	MEM	DOR	CST							
AF1a	South Africa	>32	>32	>32	>32	0.125	S	WT ^f	OXA-181	CTX-M-15	TEM-1, SHV-1	ST14	1
AF1b	South Africa	>32	>32	>32	>32	3	R	T157P	OXA-181	CTX-M-15	TEM-1, SHV-1	ST14	1
C3	Colombia	>32	>32	>32	>32	4	R	T157P	KPC-3	None	TEM-1, SHV-1	ST258	2
C4	Colombia	>32	>32	>32	>32	4	R	T157P	KPC-3	None	TEM-1, SHV-1	ST258	2
C19	Colombia	0.25	0.19	0.094	0.064	4	R	T157P	None	None	TEM-1, SHV-1	ST15	3
T2	Turkey	16	>32	>32	24	6	R	T157P	OXA-48	CTX-M-15	TEM-1, SHV-1, OXA-1	ST101	4
T3	Turkey	16	>32	>32	24	6	R	T157P	OXA-48	CTX-M-15	TEM-1, SHV-1, OXA-1	ST101	4

^a ERT, ertapenem; IPM, imipenem; MEM, meropenem; DOR, doripenem; CST, colistin.

^b R, resistant; S, susceptible.

^c ESBL, extended-spectrum β -lactamase.

^d MLST, multilocus sequence type; ST, sequence type.

^e PFGE, pulsed-field gel electrophoresis.

^f WT, wild type.

ternal standard (4). Data were compared to those obtained with the *rpsL* gene using the threshold cycle ($\Delta\Delta C_T$) method (relative), and the obtained values were then normalized against the values obtained for the susceptible isolate. Experiments were repeated three times.

Nucleotide sequence accession number. The nucleotide and protein sequences of the mutated PmrB protein were registered in GenBank under accession no. [KJ626267](#).

RESULTS

A point mutation in the *pmrB* gene of colistin-resistant *K. pneumoniae* isolates. Sequence analysis of the *pmrB* genes of all the colistin-resistant clinical isolates from our collection revealed the same single-nucleotide substitution (A to C) in six isolates (AF1b, C3, C4, C19, T2, and T3) compared to those in the *pmrB* gene sequences from *K. pneumoniae* available in GenBank (Table 2). No amino acid substitutions were identified in the PmrA, PhoP, PhoQ, and MgrB proteins in those isolates compared with sequences obtained from wild-type isolates (data not shown). This mutated PmrB protein differed from the wild type by a single threonine to proline amino acid substitution at position 157. Since a colistin-susceptible isolate, AF1a (colistin MIC of 0.125 $\mu\text{g/ml}$), recovered from the same patient from whom the colistin-resistant isolate AF1b had been recovered was available (17), its *pmrB* gene was also sequenced. A Thr residue was identified at position 157 (as for the other wild-type strains), thus reinforcing the hypothesis that Thr157Pro might play a key role in acquired resistance to colistin (Table 2).

Impact of amino acid 157 substitution on the structure of the PmrB protein. By analyzing the primary structure of the PmrB protein, amino acid 157 was identified in the PmrB domain, which is involved in the dimerization of the protein. We might speculate that the replacement of a polar by a nonpolar amino acid in this domain may have a significant impact in the dimerization process of PmrB and therefore induce constitutive activation of PmrA.

The predicted secondary structures of the two PmrB proteins (wild type and mutated) revealed that the Thr157Pro amino acid substitution significantly modified the secondary structure of the mutated protein, with an interruption of the alpha-helix (see Fig. S1 in the supplemental material).

Thr157Pro substitution in protein PmrB identified among clonally unrelated colistin-resistant *K. pneumoniae* isolates. The genetic relationship of the six colistin-resistant isolates exhibiting the same Thr157Pro substitution, together with the colistin-

susceptible isolate AF1a, was evaluated. PFGE analysis confirmed that isolates AF1a and AF1b recovered from a single patient were clonally related (17) (data not shown). In addition, isolates C3 and C4, recovered from two patients from the same hospital, were also clonally related, and isolates T2 and T3, again from two patients from the same hospital, were clonally related (data not shown). Overall, four colistin-resistant clones were identified (Table 2). Further data obtained by MLST were in accordance with the PFGE results (Table 2).

Identification of additional resistance mechanisms to broad-spectrum β -lactams. The clonally related isolates AF1a (colistin-susceptible) and AF1b (colistin-resistant) recovered from South Africa were found to produce the OXA-181 (OXA-48-like) carbapenemase (Table 2). The clonally related C3 and C4 isolates from Colombia produced carbapenemase KPC-3. Isolate C19 from Colombia, clonally unrelated compared to other isolates in this study, did not produce any carbapenemase. Finally, clonally related isolates T2 and T3 recovered from Turkey produced carbapenemase OXA-48.

Complementation experiments. To determine whether resistance to colistin might be related to the PmrB mutation, complementation experiments were performed for each clonally unrelated strain (AF1b, C3, C19, and T2). The wild-type *pmrB* gene was cloned into the high-copy-number plasmid pCR-BluntII-TOPO, which was then transformed into the colistin-resistant clinical isolates. The results of complementation showed a complete reversion to susceptibility to colistin when we transformed each of those resistant isolates with plasmid pTOPO-*pmrB* (Table 3). As expected, transformation with plasmid pTOPO-*mdh* used as a negative control did not restore the susceptibility to colistin.

Additionally, three other clonally unrelated and colistin-resistant *K. pneumoniae* for which no substitution was identified in PmrB were transformed with plasmid pTOPO-*pmrB* encoding the wild-type PmrB. MICs of colistin remained unchanged, thus showing that restoration of the susceptibility to colistin by complementation with a wild-type PmrB protein was not due to PmrB overproduction. This was also a direct proof of the involvement of the protein PmrB in the resistance pattern to colistin.

Growth rates of colistin-susceptible and colistin-resistant strains. No difference was observed between the growth of isogenic *K. pneumoniae* AF1a (colistin-susceptible) and AF1b (colistin-resistant) isolates, thus suggesting that the PmrB substitution and

TABLE 3 MIC results for colistin and polymyxin B determined by broth microdilution before and after complementation with plasmids pTOPO-*mdh* (negative control) and pTOPO-*pmrB* for the four clonally unrelated and colistin-resistant *K. pneumoniae* isolates

Isolate	MIC (μg/ml) of colistin:			MIC (μg/ml) of polymyxin B:		
	Before complementation	After complementation with pTOPO- <i>mdh</i>	After complementation with pTOPO- <i>pmrB</i>	Before complementation	After complementation with pTOPO- <i>mdh</i>	After complementation with pTOPO- <i>pmrB</i>
AF1b	16	16	1	8	8	1
C3	16	16	2	8	8	1
C19	16	16	1	8	8	2
T2	16	16	1	8	8	2

the subsequent modification of the LPS did not modify the growth properties of resistant isolates (data not shown). This observation was further confirmed by the lack of difference between the growth curves obtained for *K. pneumoniae* AF1b complemented with wild-type PmrB protein (plasmid pTOPO-*pmrB*) (colistin-susceptible) or not complemented with PmrB but with plasmid pTOPO (pTOPO-*mdh*) (colistin-resistant) (data not shown).

Thr157Pro substitution in PmrB mutation associated with overexpression of *pmrCAB* and *pmrHFIJKLM* operons. Expression levels of the *pmr* genes were measured in order to evaluate the impact of the *pmrB* mutation. RT-PCR assays were performed with isolates AF1a and AF1b, since they corresponded to likely isogenic strains. Upregulation of the expression levels of the *pmrC*, *pmrA*, *pmrB*, and *pmrK* genes was observed for AF1b compared to that for the isogenic colistin-susceptible isolate AF1a. The relative mean increases were estimated to be 170-fold for *pmrC*, 70-fold for *pmrA*, 70-fold for *pmrB*, and 40-fold for *pmrK*, whereas no significant differences in expression levels were observed with the *pmrD* and *pmrE* genes (increases of 1.9- and 1.4-fold, respectively) (Fig. 1).

DISCUSSION

Since data on the molecular bases of colistin resistance in *K. pneumoniae* are scarce, we analyzed a collection of colistin-resistant isolates of worldwide origin. Interestingly, we found six isolates harboring an unique and identical amino acid substitution in the protein PmrB. Complementation assays performed with a wild-type PmrB protein generated a complete reversion of the colistin-resistant trait. This result suggested that the PmrB substitution identified (Thr157Pro) was involved in the resistance of the clin-

ical isolates. Of note, while this work was in progress, Choi and Ko (18) identified the same mutation in a colistin-resistant *K. pneumoniae* isolate and suggested that it might be involved in colistin resistance, but no further demonstration was provided to confirm this hypothesis. The involvement of the protein PmrB in resistance to colistin correlates results obtained with colistin-resistant *Pseudomonas aeruginosa* isolates (19) or colistin-resistant *Acinetobacter baumannii* isolates (20, 21).

Furthermore, since we had two very likely isogenic isolates (one susceptible and one resistant) recovered from the same patient, this hypothesis was confirmed by the identification of this same Thr to Pro substitution at position 157 in the colistin-resistant isolate. Of note, the colistin-resistant isolate had been recovered after a selective digestive tract decontamination process based on oral colistin administration (17). Considering that a single amino acid substitution in a single protein (here PmrB) may generate resistance to colistin, we suggest that colistin-based oral decontamination has to be used with caution.

The identification of four clonally unrelated *K. pneumoniae* clinical isolates harboring the same Thr157Pro substitution in PmrB is surprising considering that they have been recovered worldwide and do not correspond to the spread of a single clone. It strongly suggests that position 157 in protein PmrB is a key position for colistin resistance. It also suggests that the Pro residue might be a key residue for resistance to colistin in *K. pneumoniae*, in contrast to recent observations made in *P. aeruginosa* or *A. baumannii* isolates from which many different amino acid substitutions occurring in PmrB have been shown to be involved in colistin resistance (18–20). Further studies, using site-directed mutagenesis in PmrB, will be performed to evaluate our hypotheses, targeting different positions, including amino acids at position 157.

Analysis of the *pmrC*, *pmrA*, and *pmrB* transcription levels by qRT-PCR in isolate AF1b carrying a *pmrB* mutation compared to those in the susceptible and isogenic AF1a indicated that this mutation leads to an overexpression of the entire *pmrCAB* operon. This result suggests that the modified PmrB induces the constitutive activation of PmrA, which in turn autoregulates (activates) the *pmrCAB* promoter, as previously demonstrated in *Salmonella* spp. (6). Expression of the *pmrC* gene, being the first gene in the *pmrCAB* operon, has been shown to lead to phosphoethanolamine addition to lipid A in *Salmonella* spp. Moreover, analysis of the level of transcription of the *pmrK* gene, which belongs to the *pmrHFIJKLM* operon, confirmed that this operon was also activated by PmrA as described previously (8). The products of this operon are responsible for 4-deoxyaminoarabinose addition and therefore modification of the LPS target. Addition of both pEtN and Ara4N to the LPS creates a more positively charged LPS molecule and thus reduces the affinity of positively charged polymyxins (6–8).

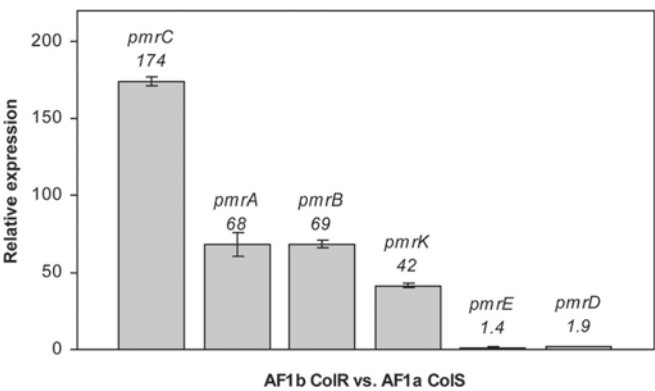


FIG 1 Relative expression levels of the *pmrC*, *pmrA*, *pmrB*, *pmrK*, *pmrE*, and *pmrD* genes in the AF1b colistin-resistant strain (ColR) compared with those in the AF1a colistin-susceptible strain (ColS). Values are the means and the standard deviations from three independent experiments.

The expression of the *pmrE* gene was not significantly modified here in the studied colistin-resistant *K. pneumoniae* isolates. Therefore, in contrast to what has been observed for *S. enterica* and *E. coli* (22), PmrA may upregulate *pmrE* expression in *K. pneumoniae*. Also, the expression of the *pmrD* gene was not significantly modified, which is different from what has been observed in *S. enterica* (23), for which the transcription of the *pmrD* gene was repressed by the PmrA protein. Those findings are in accordance with data obtained by Cheng et al. (3) showing that the *pmrD* expression is independent of PmrA/PmrB in *Klebsiella*.

In conclusion, we showed here that a specific point mutation in the PmrB protein is responsible for an upregulation of the *pmrCAB* and *pmrHFIJKLM* operons that confers resistance to colistin in *K. pneumoniae*. The identification of six clinical isolates of worldwide origin belonging to four distinct clones with this same substitution in PmrB suggests that this constitutes a clinically relevant mechanism of resistance in *K. pneumoniae*. In addition, it confirms that the PmrAB two-component system plays a major regulatory role in polymyxin B resistance in that species.

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